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Oral bioavailability and bone-sparing effects of estetrol in an osteoporosis model

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ABSTRACT

Objectives To measure the oral bioavailability of estetrol (E_4) in rats relative to its subcutaneous administration and to test the bone-sparing effect of oral E_4 compared to that of ethinylestradiol (EE).

Methods In the bioavailability study, E_4 was administered as a single dose of 0.05, 0.5 or 5.0 mg/kg orally or subcutaneously to female rats. Plasma was analyzed using an LC-MS/MS method.

The bone study was conducted in 3-month-old female rats assigned to the following seven treatment groups of ten animals each: no treatment; sham-operated + vehicle; bilaterally ovariectomized (OVX) + vehicle; OVX + E_4 0.1, 0.5, or 2.5 mg/kg/day and OVX + EE 0.1 mg/kg/day. Once-daily treatment by oral gavage was given for 4 weeks and the following measurements were performed: serum osteocalcin, bone mineral density, bone mineral content and bone mineral area of lumbar vertebrae L3–L6, peripheral quantitative computed tomography of the left tibiae and the biomechanical properties of the distal femora.

Results Oral bioavailability of E_4, relative to that of subcutaneous dosing, was 70% and above at the 0.05 and 0.5 mg/kg doses based on the $AUC_{0\text{-}t\ last}$. Subcutaneous dosing provided significantly higher E_4 levels at the 1-h time point only, and was comparable to oral dosing after 0.5, 2, 4 and 8 h.

In the bone study, E_4 dose-dependently and significantly (1) inhibited the OVX-related increase in osteocalcin levels, (2) increased bone mineral density and content, and (3) increased bone strength, all attenuated by ovariectomy. In this rat model, the relative potency of the highest dose of E_4 (2.5 mg/kg/day) was comparable to the EE dose, used as positive control.

Conclusions Estetrol exhibits high oral bioavailability in the rat, a species considered relevant for pharmacological studies that are predictive for effects on human bone. Oral administration of E_4 conveys dose-dependent bone-sparing effects of high-quality bone in estrogen-depleted OVX rats. Based on its bone-sparing effects, its oral bioavailability and its preclinical safety and efficacy profile, E_4 may be superior to other estrogens and is a potential drug for the prevention of osteoporosis in postmenopausal women.

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INTRODUCTION

Osteoporosis is a skeletal disorder characterized by compromised bone strength, predisposing patients to an increased risk of fractures[1]. Numerous studies have shown bone-sparing effects of estrogens. These effects have been documented in women experiencing type I osteoporosis due to postmenopausal estrogen depletion and, as is becoming increasingly clear, estrogen also plays a major role in bone formation and in the maintenance of bone mass in men[2,3]. Following estrogen depletion after menopause, bone mineral density (BMD) markedly decreases, at initial rates of several percent per year in the spine. The initial annual rates of BMD decreases in cortical bone, such as the midshaft radius, have been estimated to be over 1%[4,5]. Hormone therapy, consisting of either estrogen-only (estrogen replacement therapy; ERT) or estrogen in combination with a progestin to protect against estrogen-induced endometrial proliferation and cancer in women with a uterus (hormone replacement therapy; HRT) prevents these decreases in BMD[6,7]. Moreover, there is evidence that ERT/HRT reduces the fracture incidence in postmenopausal women[8], and patients with established postmenopausal osteoporosis have been successfully treated with estrogen[9,10].

In 2002, the data from the terminated Women's Health Initiative (WHI) trial showed that, when used for the prevention of postmenopausal osteoporosis in healthy women, the risks of HRT for cardiovascular disease and breast cancer seem to outweigh the benefits for fracture and colorectal cancer[11]. In 2004, the ERT arm of the WHI study was also terminated early, since the interim data showed no health benefits. The risk of stroke appeared to be increased whereas the risk of (hip) fractures was decreased[12]. The extensive debate in the scientific literature and in the lay press following this and other papers reporting data from the WHI trial is beyond the scope of this paper. A well balanced 'state-of-the-affair' Editorial addressing the status of ERT/HRT with respect to bone and cartilage after the WHI studies has been published recently[13], concluding that 'There is agreement that results from the WHI, obtained in a relatively old population of volunteers, should not be extrapolated to women during their early years after menopause, and that HRT as a preventive measure should be initiated earlier than was done in the WHI study'. This is confirmed by follow-up of women 5–15 years after short-term HRT for 2–3 years in their early postmenopausal years. Such limited HRT offers long-lasting benefits on prevention of fractures[14], cognitive impairment[15] and cardiovascular mortality and atherosclerosis[16]. Relevant for the present paper is that, in both the HRT and the ERT arms of the WHI trial, the total fracture rate, as well as the fracture rate of the femoral neck, was significantly decreased. Therefore, there is no doubt that estrogens prevent fractures; the challenge is to find safer estrogens.

Estetrol is a steroid hormone produced by the human fetal liver during pregnancy only. It was discovered by Egon Diczfalusy at the Karolinska Institute in Stockholm in 1965[17]. Structurally, estetrol is an estrogenic steroid with four hydroxyl groups (E_4). In Figure 1, the structural formulae of estrone (E_1), estradiol (E_2), estriol (E_3) and E_4 are shown. The synthesis of E_4 requires two hydroxylases (15α- and 16α-hydroxylase), expressed by the fetal liver during pregnancy only[18–20]. Substrates for endogenous E_4 biosynthesis are E_2, requiring both 15- and 16-hydroxylation, and E_3, requiring 15-hydroxylation only. Estetrol is an end-product of steroid metabolism. There is no metabolism 'backwards' to E_3 or E_2 and there are no active metabolites[21,22].

In the period between 1965 and 1985, *in vitro* and short-term *in vivo* pharmacological studies were performed to investigate the properties of E_4[21]. In a number of experimental systems, it has shown weak estrogenic effects. Competitive binding studies have revealed relatively low affinity to nuclear and cytosolic estrogen receptors[23–25]. In the rodent uterus, E_4 exerted weak agonistic effects on weight, alkaline phosphatase activity and the induction of the progesterone receptor[26,27]. Estetrol promoted growth and progesterone receptor induction in cultured estrogen-responsive human breast cancer cells (MCF-7), but over 50-fold higher concentrations were required for E_4 to elicit effects comparable to those of E_2[28]. In pregnant women, E_4 has been isolated in maternal urine as early as week 9 of gestation[29,30]. During pregnancy, E_4 has been detected at increasing concentrations in maternal plasma, reaching about 1 nanomolar concentrations in the maternal circulation at parturition and 12–19 times higher levels in the fetal circulation[30,31]. Estetrol is not present in term pregnant rats and mares (data on file). More details concerning the history of E_4 and data from

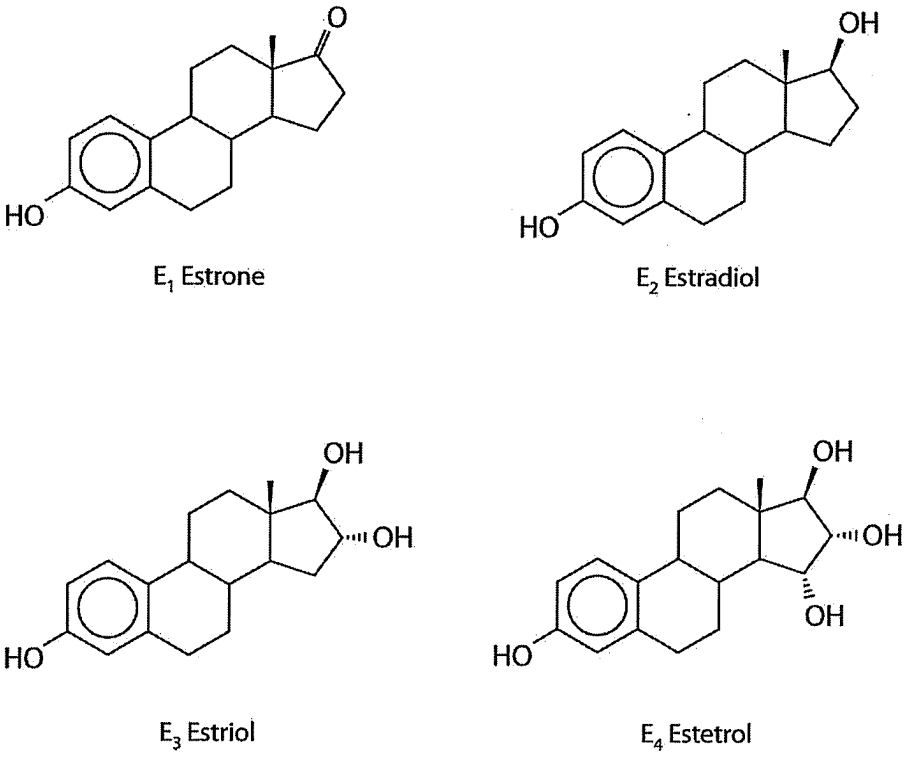

Figure 1 Structural formulae of estrone (E_1), estradiol (E_2), estriol (E_3) and estetrol (E_4)

studies in the period 1965–1985 have been summarized in a review paper[21].

Estetrol has never been proposed as a potential drug for human use and E_4 has never been administered orally to animals or by any route of administration to the human. Therefore, the very first step into the exploration of the potential medical use of E_4 was to investigate its oral bioavailability in the rat. The data are presented in this paper. When E_4 appeared to be orally bioavailable, a study, also reported in this paper, was performed to evaluate the bone-sparing effects of oral E_4 using the rat as experimental model.

Ovariectomy and estrogen replacement in ovariectomized rats have similar effects as those observed in the human skeleton[32]. The use of the ovariectomized rat model for the preclinical evaluation of drugs intended for prevention and treatment of osteoporosis is recommended by the US Food and Drug Administration (FDA)[33]. Therefore, having shown high oral bioavailability of E_4 in the rat, we chose the rat model to test the hypothesis that orally administered E_4 exerts bone-sparing effects in rats.

MATERIALS AND METHODS

Hormones

Ethinylestradiol (EE) and E_4 were purchased from Sigma (catalog number E-4876) and Steraloids Inc. (Newport, RI, USA), respectively. Estetrol (batch number G926) was 99.9% pure and did not contain any detectable E_2, as determined by HPLC-MS, NMR and DSC analysis (results not shown). Ethinylestradiol and E_4 were solubilized in arachis oil containing ≈1% ethanol (vol/vol) and were administered in a volume of 1.67 ml/kg body weight.

Rat bioavailability study

Approval for the single-dose bioavailability study was obtained from the Institutional Experimental Animal Committee. Female Sprague-Dawley rats weighing about 250 g were purchased from Harlan, (Zeist, The Netherlands) and were equipped with a permanent silastic heart catheter, as described previously[30]. Rats were allowed to recover from surgery for 5 days and were used for experiments only after regaining preoperative

body weight. Estetrol, at final doses of 0.05, 0.5 and 5.0 mg per kg body weight, was administered in a final volume of 0.5 ml arachis oil ($n = 3$ rats per dose level). For the subcutaneous treatment route, E_4 was dosed in the neck area, using a 1 ml syringe with a 20 g needle. For the oral route, E_4 was dosed intragastrically under light anesthesia by halothane/N_2O/O_2 using a plastic stomach intubator. Blood samples of 0.3 ml were collected via the heart catheter in heparinized tubes at the following times: 0.5, 1, 2, 4, 8, and 24 h after drug administration. Plasma was stored in appropriately labeled polypropylene tubes at −20°C until analysis.

Analysis of plasma E_4

Estetrol was analyzed by Xendo Laboratories (MediTech Center, Groningen, The Netherlands), using a methodology especially developed for this study. Plasma (100 µl) was extracted with 2 ml of extraction solvent (hexane : ethyl acetate, 50 : 50) after addition of internal standard and buffer. The extract was evaporated and redissolved in mobile phase and subsequently analyzed on an LC-MS/MS system (Sciex AP13000). The mobile phase was a gradient of ammonium formate buffer (10 mol/l; pH = 5) and methanol. Quantification was performed on the basis of validated calibration curves. The technical quality of the obtained results was based upon the results of concomitantly analyzed quality control samples.

Rat bone study

Three-month-old virgin female Sprague-Dawley rats (Taconic, Denmark) were maintained at 20–22°C, 55% humidity, 12-h light–dark cycle, fed a standard diet (Altomin 1324, CHR. Pedersen A/S) and tap water *ad libitum*. After an acclimatization period of 1 week, the animals were randomized according to weight into seven groups of ten each and either bilaterally ovariectomized (OVX) or sham-operated. One group of rats was sacrificed in order to obtain a baseline control. The second day after surgery, the sham-operated rats received vehicle and OVX rats received either vehicle (OVX VH); E_4 (0.1, 0.5, or 2.5 mg/kg/day) (OVX + E_4) or EE (0.1 mg/kg/day) (OVX + EE) once daily by gavage. Serum samples were collected prior to surgery and 4 weeks after surgery at the termination of the study. Femora, tibiae and lumbar vertebrae L3–L6 were isolated. The tibiae were stored in 70% ethanol at + 4°C and the femora and vertebrae were wrapped in 0.15 mol/l NaCl, 0.1% NaAzide soaked gauze and stored at + 4°C. The study was approved by The Experimental Animal Committee, The Danish Ministry of Justice, Slotsholmsgade 10, DK-1216 Copenhagen, Denmark, approval number: 1998/561-143.

Densitometry (DEXA method)

Bone mineral density (BMD), bone mineral content (BMC) and bone mineral area (BMA) of lumbar vertebrae L3–L6 were analyzed using a PIXImus densitometer and the associated software version 1.43 (Lunar Corporation, Madison, WI, USA). The area corresponding to L3–L6 was placed after initial acquisition of an image and the three parameters were measured.

Peripheral quantitative computed tomography (pQCT)

Peripheral QCT was performed on the excised left tibiae using a Stratec XCT-RM with associated software version 5.40 (Stratec Medizintechnik GmbH, Pforzheim, Germany).

Biomechanical testing

A 3-mm segment of the distal femoral metaphysis was cut directly proximal to the femoral condyle with a low-speed diamond saw under constant saline irrigation. The load was applied with a cylindrical indenter (with a flat testing face of 1.6 mm diameter) to the center of the marrow cavity on the distal face of the segment. The indenter was allowed to penetrate the cavity at a constant displacement rate of 6 mm/min to a depth of 2 mm before load reversal. The locations of maximum load, stiffness and energy absorbed were selected manually from a load displacement curve and then calculated by the instrument's software (Merlin II, Instron). Stress was calculated by dividing the maximum load by the indenter area. Maximum load (F_u in N), stiffness (S in N/mm) and energy absorbed (W in mJ) were obtained from instrument measurements and used to calculate the derived parameters: indenter cross-sectional area (CSA in mm^2) using the formula: $CSA = \pi^*(d/2)^2$, and stress (σ in N/mm^2), using the formula: $\sigma = F_u/CSA$.

Measurements of serum osteocalcin

Serum osteocalcin was determined by ELISA (Rat-MID™ ELISA, Nordic Bioscience

Diagnostics A/S, Denmark) according to the recommendations of the manufacturer. The intra- and interassay coefficients of variation were 5.0–7.7% and 3.4–5.5%, respectively.

Statistical analysis

A one-way analysis of variance (ANOVA) was used to evaluate the statistical significance between the various treatment groups in the bone study at a $p < 0.05$ level using SAS software (SAS Institute Inc., Cary, NC, USA). The ANOVA was first conducted with all treatment groups. If the test revealed significant differences, a Dunnett's multiple group comparison procedure was used to determine differences of each group to those of the untreated ovariectomized rats. Other statistical procedures comprised a Student's t test to analyze serum osteocalcin data.

RESULTS

Oral bioavailability of estetrol

Estetrol was rapidly absorbed after oral administration. As shown in Table 1, maximum plasma concentrations (C_{max}) of E_4 increased dose-dependently and were observed within 0.5–0.7 h after oral and 0.5–1.2 h after subcutaneous dosing. The estimation of the relative oral bioavailability of E_4 was primarily based on the $AUC_{0\text{-}t\ last}$ data, and was estimated at 70% or more at the 0.05 and 0.5 mg/kg dose levels by reference to the subcutaneous route of administration. The oral bioavailability at the 5.0 mg/kg dose level could not be estimated due to the large range of the individual data in this dose group and log transformation would not provide more meaningful data. A summary of the key pharmacokinetic parameters is provided in Table 1. Mean (± standard deviation, SD) E_4 plasma concentrations after oral and subcutaneous dosing with E_4 at a dose level of 0.5 mg/kg are depicted in Figure 2. The 1-h E_4 level was significantly higher after subcutaneous administration, but the 0.5-, 2-, 4- and 8-h levels were not significantly different.

Although the number of data points is rather limited, the elimination half-life of E_4 was estimated to be 2–3 h for both the 0.05 and the 0.5 mg/kg doses.

Effect of estetrol on serum osteocalcin levels

Figure 3 illustrates the effect of E_4 on serum osteocalcin levels. This figure shows the percentage changes from baseline in mean (± SD) serum levels after 4 weeks of once-daily treatment with E_4 (0.1, 0.5 or 2.5 mg/kg) or EE (0.1 mg/kg) compared to vehicle in ovariectomized rats or to sham-operated rats. Significant and favorable changes were observed in the two highest E_4 groups and in the EE group. Ovariectomized rats showed a 20% mean increase from baseline in serum osteocalcin levels. Treatment with 0.1 mg/kg/day EE for 4 weeks decreased the mean serum osteocalcin levels by 20%. Treatment with E_4 resulted in a dose-dependent attenuation of the OVX-induced increases in serum osteocalcin levels. With E_4 in a dose of 0.1 mg/kg/day, there was still an increase in osteocalcin, but less than that in the OVX group (11% vs. 20%). Both higher-dose groups of E_4 caused a significant decrease of osteocalcin (both $p < 0.01$) of 9% in the 0.5 mg/kg/day group (less than EE) and 32% in the 2.5 mg/kg/day group (more than EE).

Effect of estetrol on *ex vivo* densitometry in the lumbar spine

Table 2 presents the mean (± SD) BMC, BMA and BMD of the L3–L6 lumbar vertebrae after 4 weeks of once-daily treatment with E_4 or EE

Table 1 Mean pharmacokinetic parameters after oral or subcutaneous estetrol administration of a single dose of 0.05, 0.5 or 5.0 mg/kg to female rats

	Estetrol					
	Oral administration			*Subcutaneous administration*		
Parameter	*0.05 mg/kg* ($n=3$)	*0.5 mg/kg* ($n=3$)	*5.0 mg/kg* ($n=3$)	*0.05 mg/kg* ($n=3$)	*0.5 mg/kg* ($n=3$)	*5.0 mg/kg* ($n=3$)
C_{max} (ng/ml)	14.6	52.0	204	21.3	86.9	600
t_{max} (h)	0.7	0.7	0.5	0.7	0.5	1.2
AUC_{last} (*ng/ml)	33.9	230	1090	42.4	171	2920

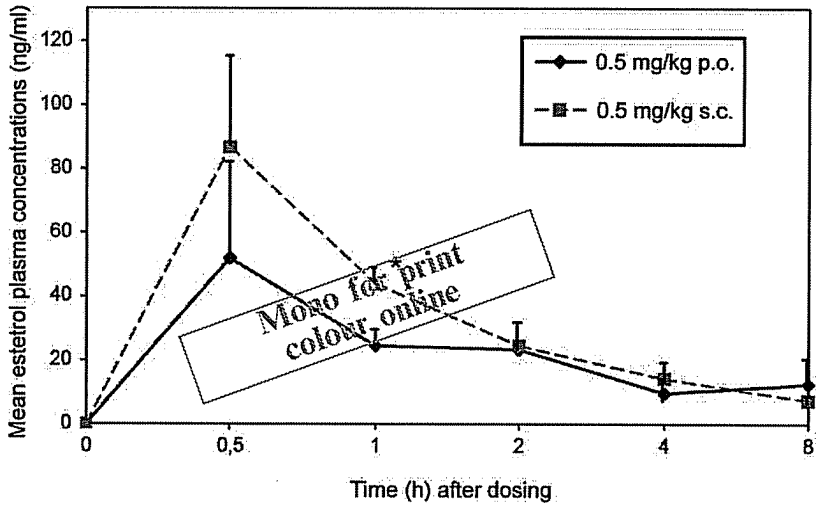

Figure 2 Mean (± standard deviation) plasma concentrations of estetrol after oral (p.o.) or subcutaneous (s.c.) administration of a single dose of 0.5 mg/kg estetrol to female rats ($n = 3$). *, Significantly different from vehicle: $p < 0.01$

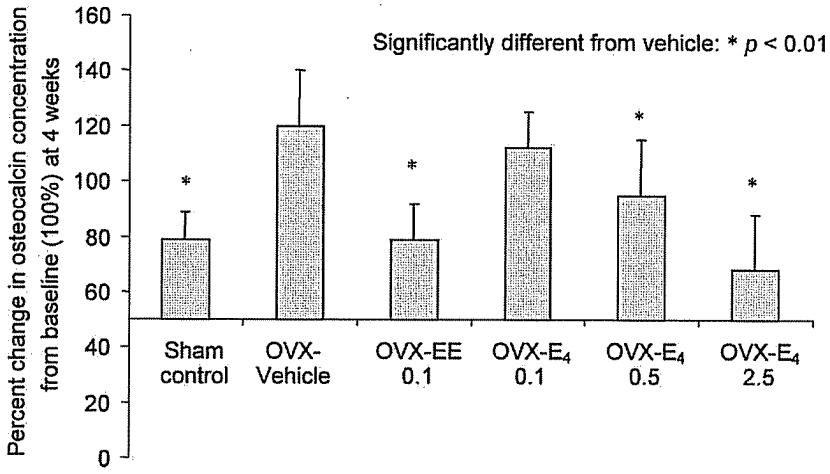

Figure 3 Mean (± standard deviation) percentage change from baseline in serum osteocalcin levels after 4 weeks of once-daily treatment with estetrol (E_4) (0.1, 0.5 or 2.5 mg/kg) or ethinylestradiol (EE) (0.1 mg/kg) compared to vehicle treatment of ovariectomized (OVX) rats and sham-operated controls

compared to vehicle in ovariectomized rats and sham-operated control animals. All three bone parameters showed the same trend. Ovariectomized vehicle-treated controls had a lower mean BMC, BMA and BMD than sham-operated controls and all three parameters increased to sham-operated levels with EE (0.1 mg/kg/day). Treatment with 0.1, 0.5 or 2.5 mg/kg/day E_4 restored BMD in a dose-dependent manner, with mean BMD values respectively 0.5%, 5.9% and 9.9% higher than the mean BMD observed for the OVX vehicle control group. A similar trend was apparent for BMC. There were no substantial differences among treatment groups for BMA. None of the treatment comparisons reached statistical significance.

Effect of estetrol on *ex vivo* pQCT in the proximal tibiae

Table 3 shows the mean (± SD) BMC and BMD of the proximal tibiae of ovariectomized rats after 4 weeks of once-daily treatment with E_4 or EE compared to vehicle in ovariectomized rats and

Table 2 Mean (± standard deviation, SD) bone mineral content (BMC), area (BMA) and density (BMD) of the L3–L6 lumbar vertebrae after 4 weeks of once-daily treatment with estetrol (E_4) (0.1, 0.5 or 2.5 mg/kg/day) or ethinylestradiol (EE) (0.1 mg/kg/day) compared to vehicle treatment of ovariectomized (OVX) rats and sham-operated controls (no significant differences)

	BMC (mg)		*BMA* (cm^2)		*BMD* (mg/cm^2)	
Treatment group	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Sham control ($n=10$)	611.6	48.5	3.35	0.13	182.5	9.0
OVX-vehicle control ($n=10$)	600.6	53.8	3.43	0.19	174.8	9.0
OVX-0.1 mg/kg/day EE ($n=10$)	615.9	46.6	3.37	0.21	183.3	11.7
OVX-0.1 mg/kg/day E_4 ($n=10$)	590.6	54.4	3.37	0.19	175.3	8.9
OVX-0.5 mg/kg/day E_4 ($n=10$)	606.1	26.0	3.36	0.13	180.7	6.2
OVX-2.5 mg/kg/day E_4 ($n=9$)	611.4	26.7	3.31	0.10	184.7	7.6

Table 3 Mean (± standard deviation, SD) bone mineral content (BMC) and density (BMD) of the proximal tibiae after 4 weeks of once-daily treatment with estetrol (E_4) (0.1, 0.5 or 2.5 mg/kg/day) or ethinylestradiol (EE) (0.1 mg/kg/day) compared to vehicle in ovariectomized (OVX) rats and sham-operated controls

	Total bone mineral				*Trabecular bone mineral*			
	Content (mg/mm)		*Density* (mg/cm^3)		*Content* (mg/mm)		*Density* (mg/cm^3)	
Treatment group	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Sham control ($n=10$)	9.36	1.08	664.07*	61.66	1.49	0.58	235.48	88.47
OVX-vehicle control ($n=10$)	8.76	0.57	606.61	44.05	1.10	0.55	169.63	84.54
OVX-0.1 mg/kg/day EE ($n=10$)	9.66	0.86	697.48**	53.11	1.81**	0.46	290.16**	67.09
OVX-0.1 mg/kg/day E_4 ($n=10$)	8.46	0.64	588.62	25.12	0.96	0.36	145.46	45.20
OVX-0.5 mg/kg/day E_4 ($n=10$)	9.74*	0.57	660.57	39.64	1.60	0.22	243.31	45.87
OVX-2.5 mg/kg/day E_4 ($n=9$)	9.61	0.67	707.11**	45.58	1.89**	0.48	309.58**	77.93

Significant vs. ovariectomized group, $*p < 0.05$; $**p < 0.01$

sham-operated controls. Ovariectomized rats showed a marked decline in BMC and BMD, as compared to sham-operated controls. The decline was observed both for total and trabecular bone, and was statistically significant for total BMD (−8.7%, $p < 0.05$). Treatment with 0.1 mg/kg/day EE restored the total and trabecular mean BMC and BMD to values similar to, or above, those observed for sham-operated controls. The increase versus OVX vehicle-treated controls was statistically significant for trabecular BMC and BMD, and for total BMD (all $p < 0.01$). Treatment with 0.1, 0.5 or 2.5 mg/kg/day E_4 dose-dependently increased total and trabecular BMC and BMD, as compared to OVX vehicle-treated controls. The increase in total BMC was statistically significant at the 0.5 mg/kg/day E_4 dose level ($p < 0.05$). The increases in trabecular BMD and BMC, and the increase in total BMD were statistically significant at the 2.5 mg/kg/day E_4 dose level ($p < 0.01$), and were comparable to the effects that were observed after treatment with EE.

A graphic presentation of the trabecular BMD of the proximal tibiae data is shown in Figure 4. Statistical significance was reached in the EE group and in the highest E_4 group.

Effect of estetrol on the indentation of the distal femora

Table 4 shows the mean (± SD) ultimate strength, maximum load, stiffness and energy at the distal femora after 4 weeks of once-daily treatment with E_4 (0.1, 0.5 or 2.5 mg/kg) or EE compared to vehicle in ovariectomized rats and sham-operated controls. The mechanical strength at the distal femora was markedly decreased following ovariectomy, as compared to sham-operated control animals, but the effect did not reach statistical significance due to the large standard deviations characteristic of this test. The mechanical strength was restored following treatment with 0.1 mg/kg EE, and was statistically significant ($p < 0.05$) for 'stiffness'. An effect of E_4 treatment relative to OVX vehicle-treated controls was apparent from

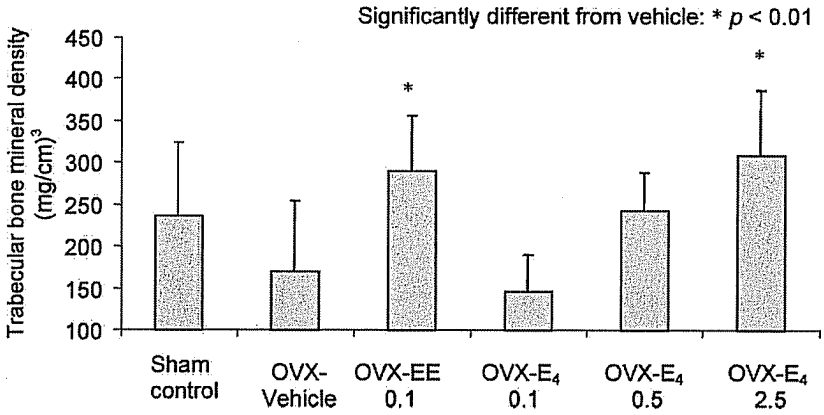

Figure 4 Mean (± standard deviation) trabecular bone mineral density (mg/cm^3) of the proximal tibiae after 4 weeks of once-daily treatment with estetrol (E_4) (0.1, 0.5 or 2.5 mg/kg/day) or ethinylestradiol (EE) (0.1 mg/kg/day) compared to vehicle treatment of ovariectomized (OVX) rats and sham-operated controls

Table 4 Mean (± standard deviation, SD) indentation testing results at the distal femur after 4 weeks of once-daily treatment with estetrol (E_4) (0.1, 0.5 or 2.5 mg/kg/day) or ethinylestradiol (EE) (0.1 mg/kg/day) compared to vehicle in ovariectomized (OVX) rats and sham-operated controls

	Ultimate strength (N/mm^2)		*Maximum load* (N)		*Stiffness* (N/mm)		*Energy* (mJ)	
Treatment group	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Sham control ($n=10$)	4.57	3.69	8.61	6.96	131.96	98.19	0.48	0.39
OVX-vehicle control ($n=10$)	1.47	1.27	2.77	2.39	42.08	38.41	0.21	0.17
OVX-0.1 mg/kg/day EE ($n=10$)	4.80	3.26	9.05	6.14	169.12*	141.29	0.53	0.66
OVX-0.1 mg/kg/day E_4 ($n=10$)	0.80	0.41	1.50	0.77	28.00	28.22	0.09	0.05
OVX-0.5 mg/kg/day E_4 ($n=10$)	3.85	2.77	7.25	5.22	132.57	108.41	0.31	0.22
OVX-2.5 mg/kg/day E_4 ($n=9$)	6.94**	4.51	13.07**	8.49	173.12*	89.33	0.68	0.53

Significant vs. ovariectomized group *$p < 0.05$; **$p < 0.01$

the dose level of 0.5 mg/kg onwards and reached statistical significance at the 2.5 mg/kg dose level with respect to increases in the ultimate strength and maximum load (both $p < 0.01$) and stiffness ($p < 0.05$). Mean (± SD) ultimate strength is depicted in Figure 5.

DISCUSSION

The data reported in the first part of this paper show that, in the rat, E_4 has a high relative oral bioavailability of above 70% compared to subcutaneous administration. The elimination half-life of 2–3 h is relatively long, since the rat liver is known to be very efficient in metabolizing steroids. These findings have at least two implications.

First, the oral bioavailability enabled once-daily oral treatment with E_4 in further studies in rats, such as the bone study reported in this paper. Second, the pharmacokinetic data obtained in the rat suggest that oral treatment with E_4 may be possible in the human too. This has been confirmed by human pharmacokinetic data[34].

The second part of this paper describes the effects of oral treatment with E_4 on bone of ovariectomized rats with EE as the positive control. The bone parameters after ovariectomy in the control group show the expected bone effects, i.e. an increase in serum osteocalcin levels, a marked decline in BMD and BMC in the lumbar vertebrae and proximal tibiae, and a decrease of mechanical strength at the distal femora. When ovariectomy is followed by 4 weeks of treatment with EE, all bone parameters under study remain similar to those observed for sham-operated controls, implying no loss of bone mass or bone strength. The effects following oral EE treatment

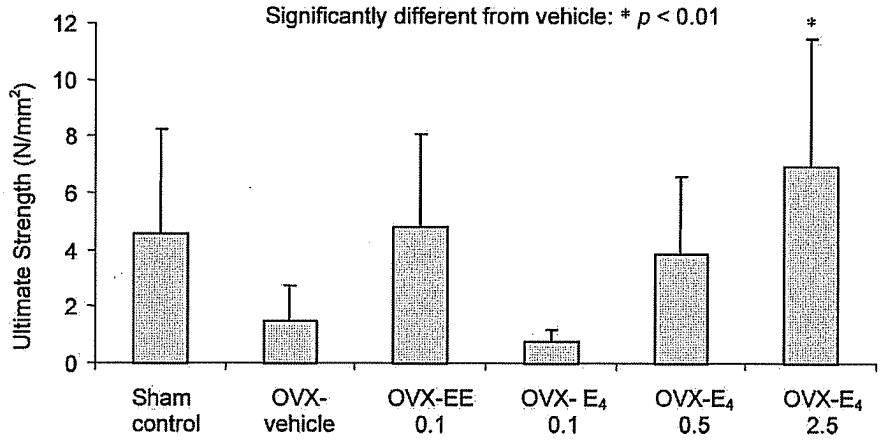

Figure 5 Mean (± standard deviation) ultimate strength (N/mm^2) at the distal femora after 4 weeks of once-daily treatment with estetrol (E_4) (0.1, 0.5 or 2.5 mg/kg/day) or ethinylestradiol (EE) (0.1 mg/kg/day) compared to vehicle in ovariectomized (OVX) rats and sham-operated controls

are similar to those observed after parenteral E_2 administration[35] and further justify the choice of oral EE as the positive control estrogen. They also confirm that our experimental model is suitable and sensitive to detect the effects of estrogen replacement on bone.

The results from the E_4 treatment groups are consistent with bone-sparing properties of the hormone. Four weeks of oral treatment with E_4 dose-dependently inhibited OVX-related increases in serum osteocalcin levels. Additionally, dose-dependent increases were apparent in BMD and BMC of the lumbar vertebrae and treatment with E_4 increased total and trabecular BMD and BMC of the proximal tibia dose-dependently.

Treatment with E_4 restored the mechanical strength of the distal femora relative to OVX vehicle-treated control rats. Especially, the highest dose of E_4 was significantly effective ($p < 0.01$) on three different criteria of bone strength. This shows that E_4 preserves bone, not just quantitatively, but also qualitatively.

The significant and dose-dependent effect of E_4 on most bone parameters tested demonstrates the preventive effect of the activity of E_4 on bone loss. Those parameters that were not statistically significant showed trends in the same favorable direction. Most likely, significance was not reached in these cases, because of the relatively low number of animals per treatment group (9–10) and/or the relatively short treatment period of 4 weeks.

Estetrol appears to be less potent than EE, since the order of increasing potency per kg/day was 0.1 mg E_4 < 0.5 mg E_4 < 0.1 mg EE < 2.5 mg E_4. This is in line with the consistent finding in other pharmacological studies in the rat of a 10–20 times higher potency of EE compared to E_4[36–39].

Taken together, the present data identify E_4 as a potential therapeutic agent for the prevention of postmenopausal osteoporosis. Safety is an essential aspect of new drugs for human treatment, especially when intended for long-term use such as the prevention of osteoporosis. As mentioned in the introduction of this paper, 2–3 years of HRT[14], as well as long-term treatment with estrogens, is effective for the prevention of fractures and HRT is related to a lower incidence of colorectal cancer[11,12,40]. However, in healthy women, those estrogens have subjective side-effects (breast tension and tenderness, weight gain, edema, nausea, vomiting, abdominal bloating, headache, irritability, depression and mood swings), may affect liver function and carry a series of rare but serious risks such as an increased incidence of venous cardiovascular disease, an increased incidence of breast cancer (primarily when combined with a progestin) and a more than doubling of all types of gallbladder disease[11,12,40–42].

Other treatment options to prevent osteoporosis in early postmenopausal women are primarily bisphosphonates and selective estrogen receptor modulators (SERMs). Those medicines have their own risk/benefit profiles that are beyond the scope of this paper. However, such alternative treatments will certainly not improve the other

symptoms of estrogen deficiency such as hot flushes and vaginal dryness, as estrogen treatment does.

Estetrol may be a potential alternative for the prevention of osteoporosis in peri- and early postmenopausal women and the data reported in this paper confirm its efficacy. Questions are, first, whether this natural estrogen has a better side-effect and safety profile compared to other estrogens, second, whether the advantages of estrogens on subjective climacteric symptoms are preserved, and, last but not least, whether E_4 has fewer subjective side-effects and is also effective in the human. The last question has to be answered by human studies. Preclinical data have been generated to study the first two questions. Those data have shown that E_4 acts as an estrogen in the vagina[36], the uterus including the endometrium[36] and the brain (effect on hot flushes[37] and ovulation inhibition[38]). Surprisingly, E_4 appeared to act pharmacologically as an estrogen antagonist in the presence of E_2 in several *in vitro* and *in vivo* models, with comparable potency to tamoxifen and ovariectomy[39]. Furthermore, *in vitro* data demonstrated little interaction between E_4 and liver function, both kinetically[22] and dynamically in the case of sex hormone binding globulin (SHBG)[43]. Since the pharmacological models used are validated and predictive for effects in the human, these preclinical findings may have the following clinical implications.

Estetrol is expected to prevent and treat vulvovaginal atrophy and vaginal dryness, dyspareunia and some types of urinary incontinence[36]; E_4 will require combination with a progestin to prevent endometrial hyperplasia and cancer[36]; E_4 may prevent and treat hot flushes[37] and, in ovulatory perimenopausal women, it is expected to inhibit ovulation, together with the progestin[38]. Most surprising were the *in vitro* and *in vivo* breast cancer studies, showing weak estrogenicity when tested as a single agent, but strong anti-estrogenicity in the presence of E_2[39], which is always the case in clinical situations. This suggests protection against breast cancer and benign breast diseases in females and possibly also in males. The concomitant progestin for the endometrium may counteract part of this protective effect[11,40]. The slow metabolism (kinetics) of E_4 explains its high oral bioavailability[34] and its long elimination half-life of 2–3 h in the rat (as shown in this study) and of 28 h in the human[34]. No binding to SHBG[43] means that all circulating E_4 is bioavailable and independent of the SHBG level, which may be influenced by the concomitant progestin. It may also implicate limited effects on other carrier proteins, angiotensinogen, lipids and hemostatic factors. The effect of E_4 on these metabolic factors can be studied easily during the further clinical development of E_4 for osteoporosis.

So far, no single non-genomic biochemical hemostatic factor has been identified that can predict the risk of venous thromboembolism (VTE) in healthy women treated with estrogens and/or progestins. The best predictor available for VTE (deep venous thrombosis, pulmonary embolism, stroke) may be the rise of SHBG. The fact that E_4 has no effect on *in vitro* SHBG synthesis[43] suggests that E_4 may have a lower procoagulant effect than other estrogens. In healthy women without arterial cardiovascular disease (CVD), estrogen treatment does not increase the CVD risk. Unpublished toxicology data on file (at Pantarhei Bioscience, The Netherlands) are also reassuring. Estetrol showed no significant binding to the human ERG channel and, in anesthetized guinea pigs, intravenous doses of up to 10 mg/kg had no effect on blood pressure, heart rate or the ECG.

The increased incidence of all types of gallbladder disease (stones, infection, malignancy) has obtained remarkably little attention as a complication of estrogen use. Observational studies indicate a greater than two-fold risk of all biliary tract conditions related to estrogen therapy, including the use of oral contraceptives and postmenopausal estrogens[41]. This side-effect of estrogen use has also been well documented in the prospective, randomized controlled Women's Health Initiative study[42]. Contrary to other estrogens, excretion of E_4 does not only occur via the liver, but also to a significant extent via the kidneys[44,45]. The lower exposure of the biliary tract to estrogens when using E_4 may result in a lower incidence of gallbladder disease.

There are additional reasons to expect that E_4 will be safe for the prevention of osteoporosis. First of all, E_4 is a natural steroid, present during human pregnancy at rising levels. At term, the fetus synthesizes about 3 mg E_4 per day[45] and the daily fetal exposure to E_4 is comparable to treatment of adult women with an oral dose of 50–55 mg E_4 per day[34]. Apparently, this is safe for the fetus. Second, E_4 has exclusive affinity for both the estrogen-α and -β receptors and not for any other steroid receptor or 124 other drug targets tested[22]. Third, in an extensive pharmacological study program in rats[36–39], including the study reported in this paper, with E_4 doses of up to 3 mg/kg/day for 8 weeks and 10 mg/kg/day for

4 weeks, no animals died and no side-effects occurred. Fourth, in unpublished toxicity studies (data on file), no acute toxicity was observed in a 5-day study in rats with a maximum dose of 350 mg/kg/day. The Ames mutagenicity test was negative. Finally, in human studies, doses of E_4 of up to 40 mg/day for 28 days in postmenopausal women have not shown any major side-effects (data from ongoing study by Pantarhei).

In summary, the E_4 data from the literature[21] as well as the data reported and referred to in this paper raise minimal, if any, safety concerns for the use of E_4 in humans. However, long-term data will be required for a final judgement.

Altogether, it seems worthwhile to consider the use of E_4 as bone-sparing treatment for physiological bone loss related to menopause as a feasible option. Further development will require proof-of-concept studies focusing first on bone mineral density and, when positive, on fracture rate.

CONCLUSIONS

As documented in this paper, the human fetal estrogenic steroid E_4 has a remarkably high oral bioavailability in the rat, a species considered relevant for pharmacological studies that are predictive for effects on human bone.

This paper further documents the bone-sparing effects of orally administered E_4 by evaluating a variety of relevant endpoints. Estetrol prevented the ovariectomy-related increases in serum osteocalcin in a dose-dependent manner; it dose-dependently restored bone mineral density and bone mineral content in the lumbar spine of ovariectomized rats. Likewise, E_4 restored total and trabecular bone mineral density and bone mineral content of the proximal tibiae and prevented the ovariectomy-related decrease in mechanical strength of the distal femora.

Based on its bone-sparing effects, its oral bioavailability and its preclinical safety and efficacy profile, E_4 may be superior to other estrogens and is a potential drug for the prevention of osteoporosis in postmenopausal women.

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